ZooKeys 540: 193–209 (2015)
doi: 10.3897/zookeys.540.9649
http://zookeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
ZooKeys
Launched to accelerate biodiversity research

# Characterisation of the chemical profiles of Brazilian and Andean morphotypes belonging to the *Anastrepha fraterculus* complex (Diptera, Tephritidae)

Lucie Vaníčková[1,2], Radka Břízová[2,3], Antonio Pompeiano[4], Luana Lima Ferreira[1], Nathaly Costa de Aquino[1], Raphael de Farias Tavares[1], Laura D. Rodriguez[5], Adriana de Lima Mendonça[1], Nelson Augusto Canal [5], Ruth Rufino do Nascimento[1]

**1** *Laboratório de Ecologia Química, Instituto de Química e Biotecnologia, Universidade Federal de Alagoas, Av. Lourival de Melo Mota, s/n, Tabuleiro, CEP 57072-970, Maceió, AL, Brazil* **2** *Institute of Organic Chemistry and Biochemistry ASCR, v.v.i., Flemingovo nám. 2, CZ-166 10 Prague 6, Czech Republic* **3** *Institute of Chemical Technology in Prague, Technická 5, CZ-166 28 Prague 6, Czech Republic* **4** *Laboratory of Plant Physiology, Centre of Agricultural Sciences, Federal University of Alagoas, Maceió, AL, Brazil* **5** *Universidad del Tolima, Barrio Santa Helena Parte Alta, A.A. 546, CP 730006299, Ibague, Colombia*

Corresponding author: *Lucie Vaníčková* (luci.vanickova@gmail.com)

Academic editor: *M.T. Vera* | Received 20 March 2015 | Accepted 3 July 2015 | Published 26 November 2015

*http://zoobank.org/5B3C9301-F455-4650-87E8-A17A2F2A6CDD*

**Citation:** Vaníčková L, Břízová R, Pompeiano A, Ferreira LL, de Aquino NC, Tavares RF, Rodriguez LD, Mendonça AL, Canal NA, do Nascimento RR (2015) Characterisation of the chemical profiles of Brazilian and Andean morphotypes belonging to the *Anastrepha fraterculus* complex (Diptera, Tephritidae). In: De Meyer M, Clarke AR, Vera MT, Hendrichs J (Eds) Resolution of Cryptic Species Complexes of Tephritid Pests to Enhance SIT Application and Facilitate International Trade. ZooKeys 540: 193–209. doi: 10.3897/zookeys.540.9649

## Abstract

Fruit fly sexual behaviour is directly influenced by chemical and non-chemical cues that play important roles in reproductive isolation. The chemical profiles of pheromones and cuticular hydrocarbons (CHs) of eight fruit fly populations of the Andean, Brazilian-1 and Brazilian-3 morphotypes of the *Anastrepha fraterculus* cryptic species complex originating from Colombia (four populations) and Brazil (four populations) were analysed using two-dimensional gas chromatography with mass spectrometric detection. The resulting chemical diversity data were studied using principal component analyses. Andean morphotypes could be discriminated from the Brazilian-1 and Brazilian-3 morphotypes by means of male-borne pheromones and/or male and female CH profiles. The Brazilian-1 and Brazilian-3 morphotypes were found to be monophyletic. The use of chemical profiles as species- and sex-specific signatures for cryptic species separations is discussed.

Copyright *Lucie Vaníčková et al.* This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Keywords**
Cryptic species, chemotaxonomy, GC×GC/MS, PCA

## Introduction

Communication is a crucial process for both intra- and interspecific interactions (Kroiss 2008). Chemical signals are probably the oldest form of communication in living organisms, and insects constitute one group of animals that rely heavily on chemical communication signals (Levine and Millar 2009). Unique messages are created by varying the structure of the chemicals that comprise a message and by combining these chemicals in blends with different ratios (Jallon 1984, Millar 2000). Over time, the components of the messages have been tuned up by natural selection according to function within the context in which they evolved (Levine and Millar 2009, Vaníčková 2012).

The South American fruit fly, *Anastrepha fraterculus* (Wiedemann 1830) (Diptera: Tephritidae) constitutes a cryptic species complex with different degrees of pre- and postzygotic isolation (reviewed by Vaníčková et al. 2015a). Eight taxonomically distinct morphotypes have been recognized thus far; these are the Andean, Brazilian-1, Brazilian-2, Brazilian-3, Ecuadorian, Mexican, Peruvian and Venezuelan morphotypes (Hernández-Ortiz et al. 2004, 2012, 2015). Recent studies have shown that the Andean, Brazilian-1, Brazilian-3, Mexican, and Peruvian morphotypes have varying degrees of sexual incompatibility (Vera et al. 2006, Cáceres et al. 2009, Segura et al. 2011, Rull et al. 2013, Devescovi et al. 2014). *Anastrepha fraterculus* exhibits a complex mating system, which involves auditory, visual and chemical signals (Morgante et al. 1983, Aluja 1994). Males of this species form leks on host or non-host trees to release volatile compounds, which serve at first to attract other males and subsequently to attract conspecific females. These chemical mixtures therefore act as aggregation as well as sexual pheromones. Differences in the *A. fraterculus* male-borne volatiles produced by flies from geographically distinct populations from Argentina, Brazil and Peru were previously described (Lima et al. 2001, Cáceres et al. 2009, Břízová et al. 2013, Lima-Mendonça et al. 2014). Recently, chemical and electrophysiological analyses have shown that six compounds of the volatile mixture produced by males of *A. fraterculus* from northeastern Brazil are antennaly active in conspecific females (Milet-Pinheiro et al. 2015). The attractiveness of conspecific females in laboratory and semi-field bioassays for a synthetic mixture of the six components to conspecific females is similar to the attractiveness of male headspace samples (Milet-Pinheiro et al. 2015).

It is thought that tephritid fruit flies use contact pheromones in the last phase of courtship when a female briefly touches a male with its proboscis or front legs (Morgante et al. 1980, Vaníčková et al. 2014, Vaníčková et al. 2015b). Contact or short-range pheromones are relatively non-volatile, nonpolar compounds called cuticular hydrocarbons (CHs), which are synthesized from fatty acid precursors and deposited on an insect's cuticle. Essentially, they serve as protection against desiccation. They also

play a crucial role in communication and sex, species, or reproductive status recognition (Blomquist and Bagnères 2010, Kather and Martin 2012). Due to their species-specificity, CH profiles are widely used for cryptic species determination, as documented in diverse insect families such as Termitoidae (Haverty et al. 2000), Miridae (Gemeno et al. 2012), Tephritidae (Vaníčková et al. 2014, Vaníčková et al. in press), Drosophilidae (Oliveira et al. 2011, Jennings et al. 2014), and many others.

In *A. fraterculus* age- and sex-dependent CHs production has been investigated in one Argentinean population (Vaníčková 2012, Vaníčková et al. 2012b). Males of *A. fraterculus* from this population produce a set of unsaturated CHs, not present in female body washes, suggesting these compounds may function as contact pheromones in chemical communication. A recent study on intraspecific variation of male CH profiles from six populations of the *A. fraterculus* cryptic species complex, ranging from Argentina to Mexico, has shown that the chemical signatures are specific to the putative species and therefore may be used for identification of particular morphotypes within the complex (Vaníčková et al. in press). In the same study, female CH profiles of Brazilian-1, Peruvian and Mexican morphotypes revealed qualitative and quantitative differences in CH composition. Nevertheless, a detailed study using more populations of the same *A. fraterculus* morphotype from different regions is necessary for further evaluation of the male pheromone and CH profiles as potential chemotaxonomic markers in species differentiation. Furthermore, information on the variability of female CH profiles within putative species of the *A. fraterculus* complex needs to be evaluated.

The present work aims to (i) clarify differences in the composition of male-borne volatiles among eight different populations belonging to three morphotypes (Andean, Brazilian-1 and Brazilian-3) of the *A. fraterculus* cryptic species complex; (ii) evaluate the potential use of male CH profiles for *A. fraterculus* identification of the Andean, Brazilian-1 and 3 morphotypes; (iii) investigate divergence in female CH profiles of the Andean and Brazilian morphotypes.

## Methods

### Insects

Eight laboratory populations, previously analysed for the morphotype identification by Hernández-Ortiz et al. (2004, 2012, 2014 unpublished data), originated from unique collections (for more detail see Table 1). After eclosion, the insects were separated by sex and put into plastic chambers (30 × 20.5 × 16 cm). The flies were fed an artificial diet (Sobrinho et al. 2009). Brazilian morphotypes were reared in the Chemical Ecology Laboratory at the Universidade Federal de Alagoas (Maceio, Brazil). The temperature of the insectarium was 25 °C, relative humidity was 60%, and the photoperiod was 14:10 light:dark. The Andean morphotypes were kept in the entomological laboratories at the Universidad del Tolima (Ibague, Colombia). They were reared at 22 °C; relative humidity was 70%, and the photoperiod was 12:12 light:dark.

**Table 1.** Geographical location of the sampled populations of *Anastrepha fraterculus* in Brazil and Colombia and the analyses of their cuticular hydrocarbons (CH) and male-borne pheromones.

| Country, State | Population | Code | Alt. [m] | Latitude | Longitude | Morphotype† | CH analyses | Pheromone analyses |
|---|---|---|---|---|---|---|---|---|
| Brazil, RS | Pelotas‡ | PEL | 7 | 29°28.19'S | 50°37.03'W | Brazilian-1 | + | + |
| Brazil, RS | Bento Gonçalves‡ | BEN | 690 | 29°17.08'S | 51°51.89'W | Brazilian-1 | + | + |
| Brazil, SC | São Joaquim§ | SAO | 1360 | 28°17.38'S | 49°55.54'W | Brazilian-1 | + | + |
| Brazil, AL | Alagoas\| | AL | 16 | 10°08.01'S | 36°10.34'W | Brazilian-3 | + | + |
| Colombia | Ibague¶ | IBA | 1580 | 04°26.20'N | 75°13.55'W | Andean | n.a. | + |
| Colombia | Sibundoy¶ | SIB | 2136 | 01°20.33'N | 76°91.92'W | Andean | + | + |
| Colombia | Duitama¶ | DUI | 2569 | 05°49.50'N | 73°04.49'W | Andean | + | + |
| Colombia | Cachipay¶ | CAC | 1850 | 04°45.27'N | 74°23.02'W | Andean | + | n.a. |

†Morphotypes were identified by Dr. Hernández-Ortiz. Institutions and laboratories which provided the particular populations: ‡EMBRAPA-Empresa Brasileira de Pesquisa Agropecuária, Brazil; §UFB- Universidade Federal de Bahia, Brazil; |UFAL-Laboratory of Chemical Ecology at Universidade Federal de Alagoas, Brazil; ¶UT-Universidad del Tolima, Colombia. n.a. – samples were not avaliable for the analyses.

## Male-borne volatile collection

For the male-volatile collection following populations were available: three Colombian (Duitama, Ibague, Sibundoy) and four Brazilian (Alagoas, Bento Gonçalves, Pelotas, São Joaquim) (Table 1). To obtain male-borne volatiles, procedures described by Břízová et al. (2013) and Milet-Pinheiro et al. (2015) were used. Groups of 20 sexually mature virgin males of *A. fraterculus* were placed in a glass desiccator (180 mm high; 200 mm diameter), and volatiles were collected using dynamic headspace methods. The inlet of the desiccator was modified by the addition of an inlet tube containing SuperQ® (100 mg; Chromapack) to adsorb the released volatiles. The volatiles were then collected using an air pump (Resun® AC 2600) coupled to a flow meter (Supelco®) and absorbed on the SuperQ® filter. The air flow through the filter was 500 mL $min^{-1}$ for 24 h. The volatiles trapped in the filter were then eluted with 500 μL of redistilled trace analysis grade hexane (Sigma-Aldrich, Brazil). The samples were stored in 2 mL vials, which were kept in the freezer (-5 °C) until chemical analyses. Ten replicate samples of volatiles were collected for each of the study populations.

## Extraction of cuticular hydrocarbons

For the extraction of CHs following populations were available: three Colombian (Duitama, Cachipay, Sibundoy) and four Brazilian (Alagoas, Bento Gonçalves, Pelotas, São Joaquim) (Table 1). CHs of 20-days-old virgin males ($N$ = 10) and females ($N$ = 10) were extracted from the study populations following previously described methods (Vaníčková et al. 2012b, Vaníčková et al. 2014, Vaníčková et al. in press). 1-Bromdecane (Sigma-Aldrich, Czech Republic) was added as an internal standard for quantification (10 ng per 1 μL of the extract). Each extract was concentrated to approximately 100 μL under a constant flow of nitrogen and stored in a freezer (-5 °C) until analysis.

## Chemical analyses

Two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC/MS) was used for the quantification and identification of male-borne volatiles and CH profiles. Identical conditions were used for the analyses of all samples and for chromatographic data evaluation, as described in previous studies on *A. fraterculus* male pheromones (Břízová et al. 2013, Milet-Pinheiro et al. 2015) and CHs (Vaníčková 2012, Vaníčková et al. 2012b, Vaníčková et al. in press). Standards of *n*-alkane ($C_8$–$C_{38}$; Sigma-Aldrich, Czech Republic) were co-injected with authentic samples to determine the retention indices (*RI*) of the analytes. Compounds were identified by comparison of their mass spectra fragmentation patterns, *RI* and authentic synthetic standards when available (Van Den Dool and Kratz 1963, Carlson and Yocom 1986, Vaníčková 2012, Vaníčková et al. 2012a, Břízová et al. 2013, Milet-Pinheiro et

al. 2015, Vaníčková et al. 2014, Vaníčková et al. in press). Detailed chemical identification of the CHs has been published previously (Vaníčková et al. 2012b, Vaníčková et al. in press).

### Chemicals

Except for (*S,S*)-(-)-epianastrephin, which was provided by Prof. Jim Nation (University of Florida, Gainesville, USA), all chemicals were purchased either from Sigma-Aldrich, Brazil [α-pinene, limonene, (*Z*)-3-nonen-1-ol] or from Penta, USA [(*E,Z*)-3,6-nonadien-1-ol]. Chemicals were > 95% pure, based on the results from capillary gas chromatography.

### Statistics

The relative peak areas of seven male-borne volatile compounds and forty-eight male and female CHs (as identified by the GC×GC/MS in the deconvoluted total-ion chromatogram mode) were calculated for each replicate of the study populations.

The differences in the chemical composition of the samples from study populations were analysed by principal component analysis (PCA). Prior to PCA, peak areas were subjected to logarithmic transformation; intraspecific scaling was performed by dividing each species score by its standard deviation; the data were centred by species' scores. In PCA analyses, hierarchical clustering based on Pearson correlation showed that populations with similar chemical profiles cluster together.

A heat map was used to visualize male-borne volatiles organized as matrices. The heat map performed two actions on a matrix. First, it reordered the rows and columns so that rows and columns with similar profiles were closer to one another, causing these profiles to be more visible to the eye. Second, each entry in the data matrix was displayed as a colour, making it possible to view the patterns graphically. Dendrograms were created using correlation-based distances and the Ward method of agglomeration was used in the present analysis (Key 2012). All computations were performed with R 3.1.2 (R Core Team 2014), and the R packages *FactoMineR* (Husson et al. 2015), and *gplots* (Warnes et al. 2015) were used.

## Results

### Male-borne volatiles

Significant quantitative differences ($P < 0.05$) were found in the male-borne volatiles, namely α-pinene, limonene, (*Z*)-3-nonen-1-ol, (*E,Z*)-3,6-nonadien-1-ol, (*Z,E*)-α-farnesene, (*E,E*)-α-farnesene and epianastrephin, among the investigated populations

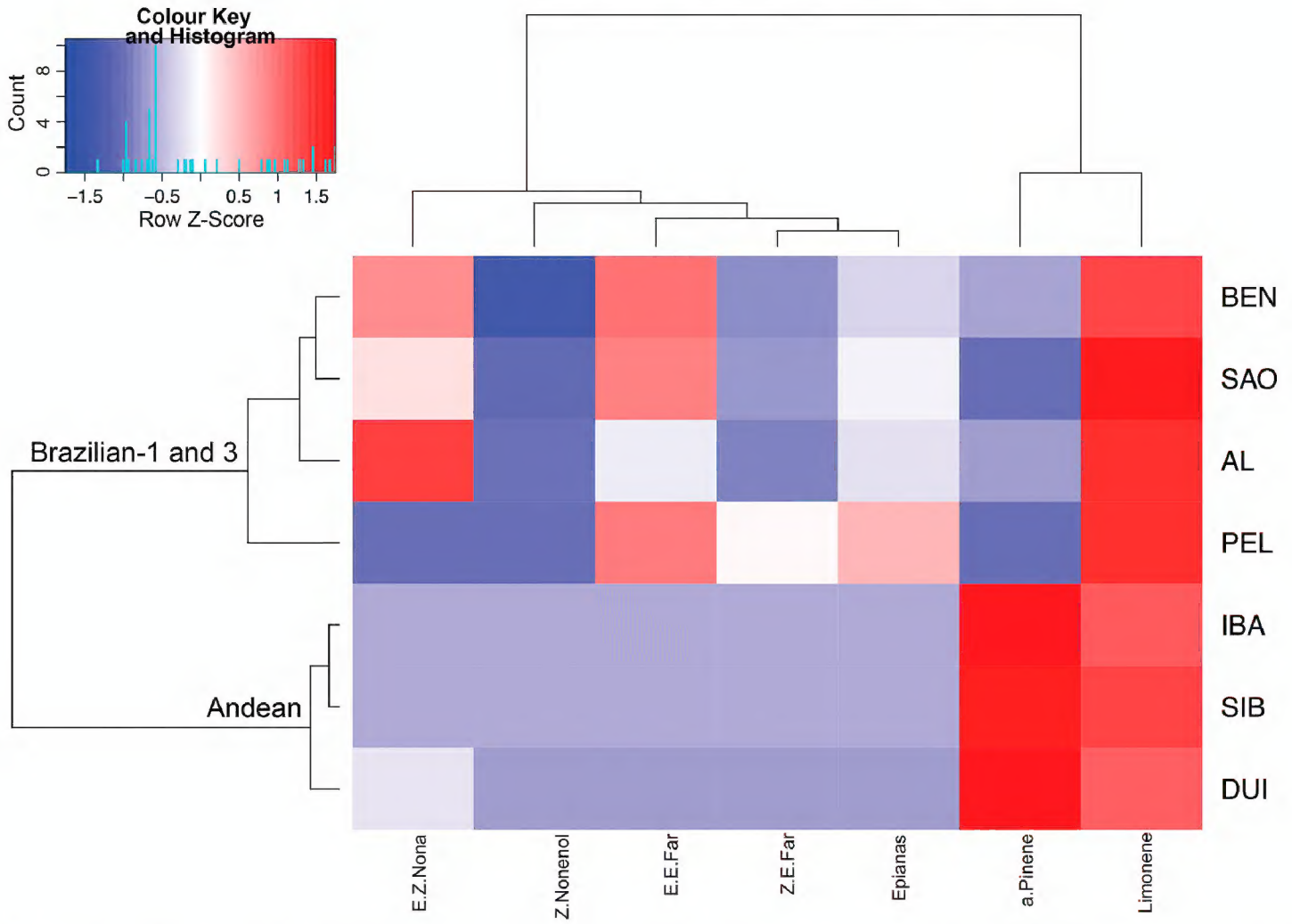


**Figure 1.** Heat map of seven male-borne volatiles (columns) identified by GC×GC/MS analyses in seven populations (rows) of the *Anastrepha fraterculus* cryptic species complex. The dendrograms were created using correlation-based distances and the Ward method of hierarchical clustering ($P < 0.05$). Key: AL – Alagoas, AL, Brazil; BEN – Bento Gonçalves, RS, Brazil; DUI – Duitama, Colombia; IBA – Ibague, Colombia; PEL – Pelotas, RS, Brazil; SAO – São Joaquim, SC, Brazil; SIB – Sibundoy, Colombia. Epianas – Epianastrephin; Z.E.Far – (*Z*,*E*)-α-farnesene; E.E.Far – (*E*,*E*)-α-farnesene; Z.Nonenol – (*Z*)-3-nonen-1-ol; E.Z.Nona – (*E*,*Z*)-3,6-nonadien-1-ol; a.Pinene – α-pinene.

of *A. fraterculus*. A heat map was constructed to visualize the relative proportions of the seven volatiles in each of the populations (Figure 1). Colombian populations (DUI, IBA, SIB) have similar proportions of the seven compounds, whereas the Brazilian populations (AL, BEN, PEL, SAO) have diverse volatile profiles. In the compound dendrogram, monoterpenes (α-pinene, limonene) formed one cluster, while sesquiterpenes [(*Z*,*E*)-, (*E*,*E*)-α-farnesenes], unsaturated alcohols [(*Z*)-3-nonen-1-ol, (*E*,*Z*)-3,6-nonadien-1-ol] and a lactone (epianastrephin) grouped in a second cluster. The most abundant volatile among all the populations was limonene. (*Z*)-3-Nonen-1-ol was the least abundant compound in the study strains. α-Pinene was more abundant in the Andean morphotype (DUI, IBA, SIB) than in the Brazilian morphotypes (AL, BEN, PEL, SAO). The relative proportion of (*E*,*Z*)-3,6-nonadien-1-ol varied the most between populations.

The PCA analyses of GC×GC/MS data showed the Andean morphotype formed one cluster while Brazilian-1 and Brazilian-3 morphotypes formed another cluster (Figure 2, Hierarchical clustering). The first two dimensions represented 85% of

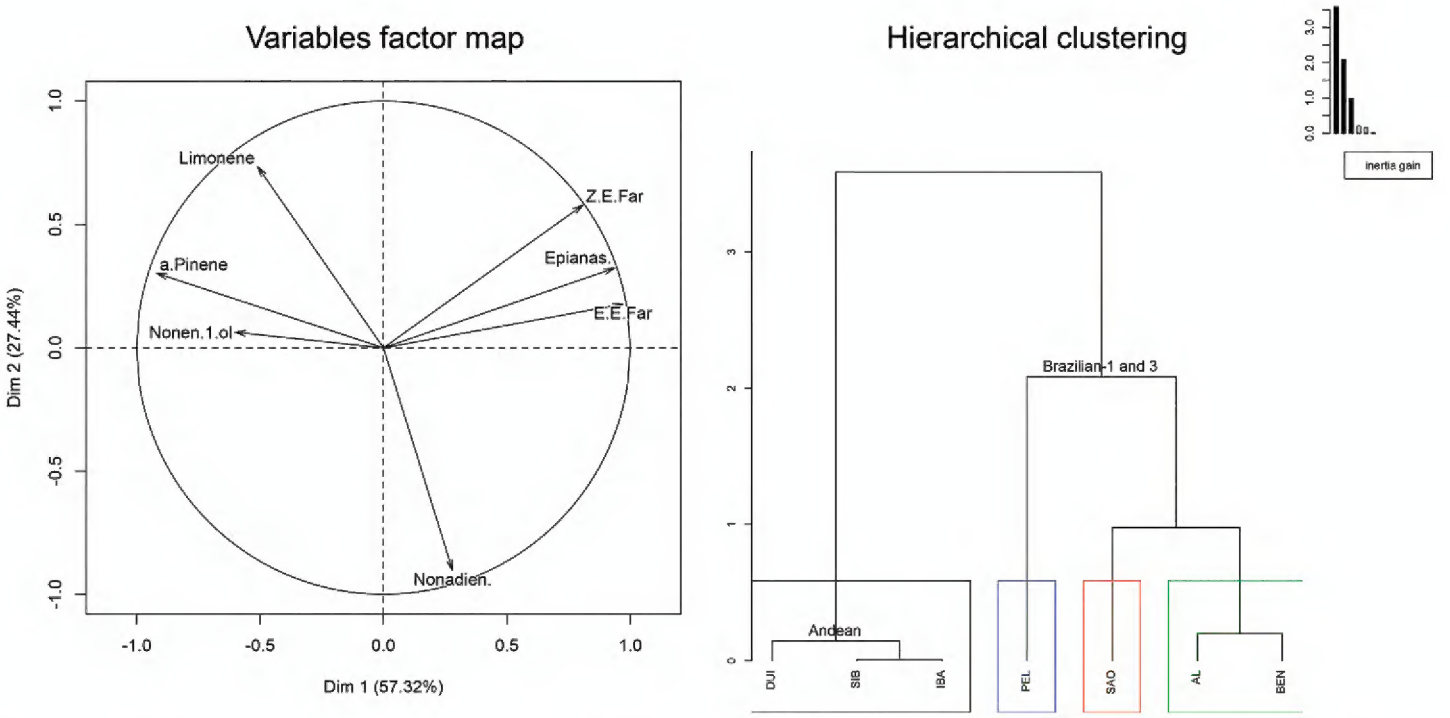


**Figure 2.** Principal component analyses (PCA) of transformed GC×GC/MS data of seven male-borne volatiles produced by groups of 20 sexually mature individuals from seven populations of the *Anastrepha fraterculus* cryptic species complex. Variables factor map represents projection of variables on the plane defined by the first two principal components. Hierarchical clustering is score plot describing the populations and their clustering. Key: AL – Alagoas, AL, Brazil; BEN – Bento Gonçalves, RS, Brazil; DUI – Duitama, Colombia; IBA – Ibague, Colombia; PEL – Pelotas, RS, Brazil; SAO – São Joaquim, SC, Brazil; SIB – Sibundoy, Colombia. Epianas. – Epianastrephin; Z.E.Far – (*Z*,*E*)-α-farnesene; E.E.Far – (*E*,*E*)-α-farnesene; Nonen.1.ol – (*Z*)-3-nonen-1-ol; Nonadien. – (*E*,*Z*)-3,6-nonadien-1-ol; a.Pinene – α-pinene. Colored boxes indicate particular clusters.

the total variance. The monoterpenes α-pinene and limonene and (*Z*)-3-nonen-1-ol were specific to the Andean morphotype, whereas sesquiterpenes (*Z*,*E*)- and (*E*,*E*)-α-farnesene, the lactone epianastrephin and (*E*,*Z*)-3,6-nonadien-1-ol were typical for the Brazilian morphotypes (Figure 2, Variables factor map). The three populations representing the Andean morphotype (DUI, IBA, SIB) grouped together forming the first cluster (Figure 2, Hierarchical clustering) whereas the second cluster was formed by the Brazilian populations representing the Brazilian-1 (BEN, PEL, SAO) and Brazilian-3 (AL) morphotypes.

## Male CH profiles

Forty-eight male CHs, including 19 linear *n*-alkanes (A1-19), 11 methylbranched alkanes (B1-11), 11 alkenes (C1-11), and 7 alkadienes (D1-7), were evaluated by PCA for the possible use in the identification of Andean (CAC, DUI, SIB), Brazilian-1 (BEN, PEL, SAO) and Brazilian-3 (AL) morphotypes (Figure 3). The first two PCA dimensions accounted for 55% of the total variance. The populations clustered in two main groups, and each group was composed of two clusters. Clusters one and two were formed by Andean morphotype populations (DUI and CAC, SIB, respectively),

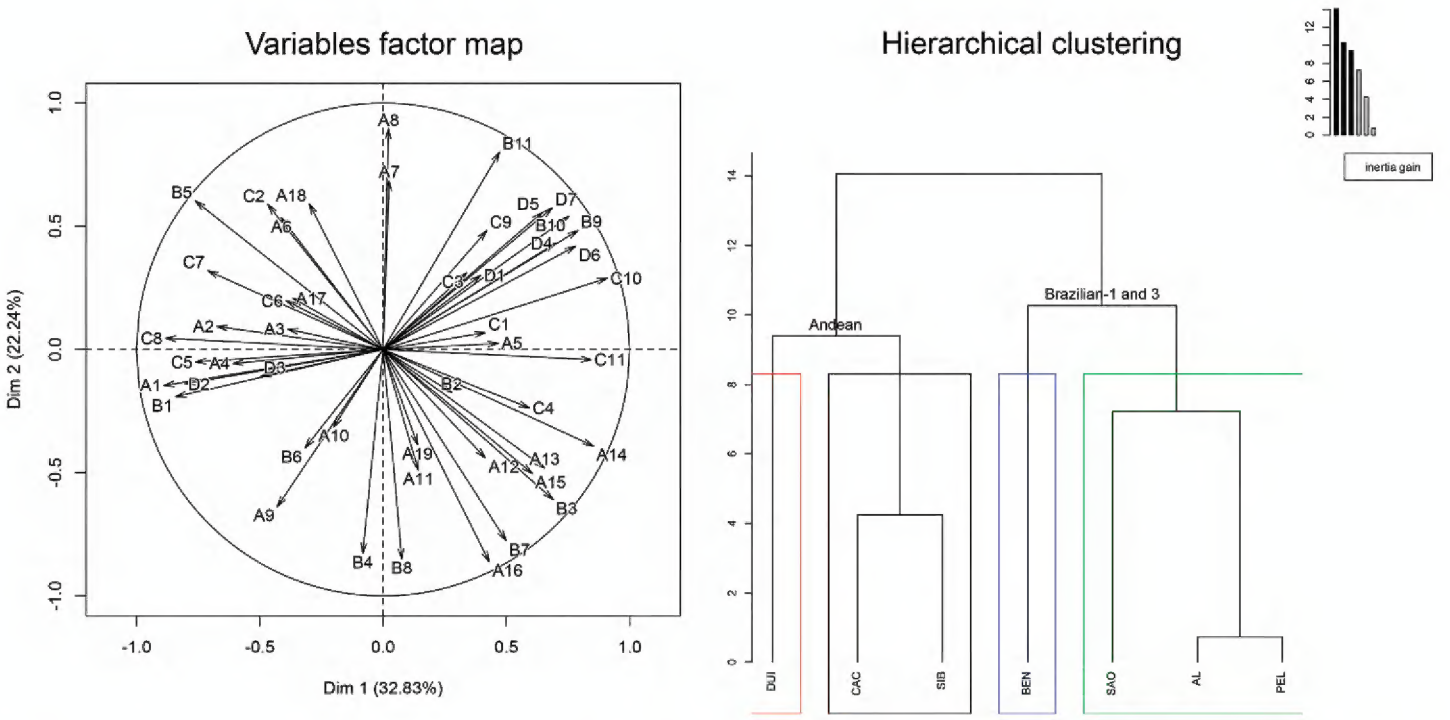


**Figure 3.** Principal component analyses (PCA) of transformed GC×GC/MS data of 48 male CHs from seven populations of the *Anastrepha fraterculus* cryptic species complex. Variables factor map represents projection of variables on the plane defined by the first two principal components. Hierarchical clustering is score plot describing the populations and their clustering. Key: AL – Alagoas, AL, Brazil; BEN – Bento Gonçalves, RS, Brazil; CAC – Cachipay, Colombia; DUI – Duitama, Colombia; PEL – Pelotas, RS, Brazil; SAO – São Joaquim, SC, Brazil; SIB – Sibundoy, Colombia. A1–19 – *n*-alkanes; B1–11 – methyl-branched hydrocarbons; C1–11 – alkenes; D1–7 – alkadienes. Colored boxes indicate particular clusters.

whereas clusters three and four consisted of Brazilian morphotypes populations (BEN and AL, PEL, SAO; Figure 3, Hierarchical clustering). Linear *n*-alkanes (*n*-nonacosane A14, *n*-hentriacontane A16), methylbranched hydrocarbons (3-methylheptacosane B3, methylheptatriacontane B11) and 11-tritriacontene (C10) were responsible for the formation of the Brazilian morphotype clusters (AL, BEN, PEL, SAO), whereas *n*-dodecane (A1) and the mix of odd methylbranched hydrocarbons (9-/11-/13-methylnonacosane B5) were characteristic for the Andean group (Figure 3, Variables factor map, Suppl. material 1).

## Female CH profiles

Female CH profiles from study populations consisted of 48 saturated and unsaturated compounds with chain lengths ranging from 12-38 carbons. In female body washes, unsaturated male-specific CHs were absent, namely 7-heneicosene, 7-docosene, 7-tricosene and 7-pentacosene. In the PCA analyses the populations segregated into two main groups (Figure 4, Hierarchical clustering). The three Colombian populations (CAC, DUI, SIB) grouped together forming one cluster. The second cluster was formed by the four Brazilian populations (AL, BEN, PEL, SAO) (Figure 4, Hierarchical clustering). The compounds responsible for this separation were *n*-docosane (A7), methylbranched hydrocarbons (2-methyloctacosane B4, 3-methylnonacosane B7) and

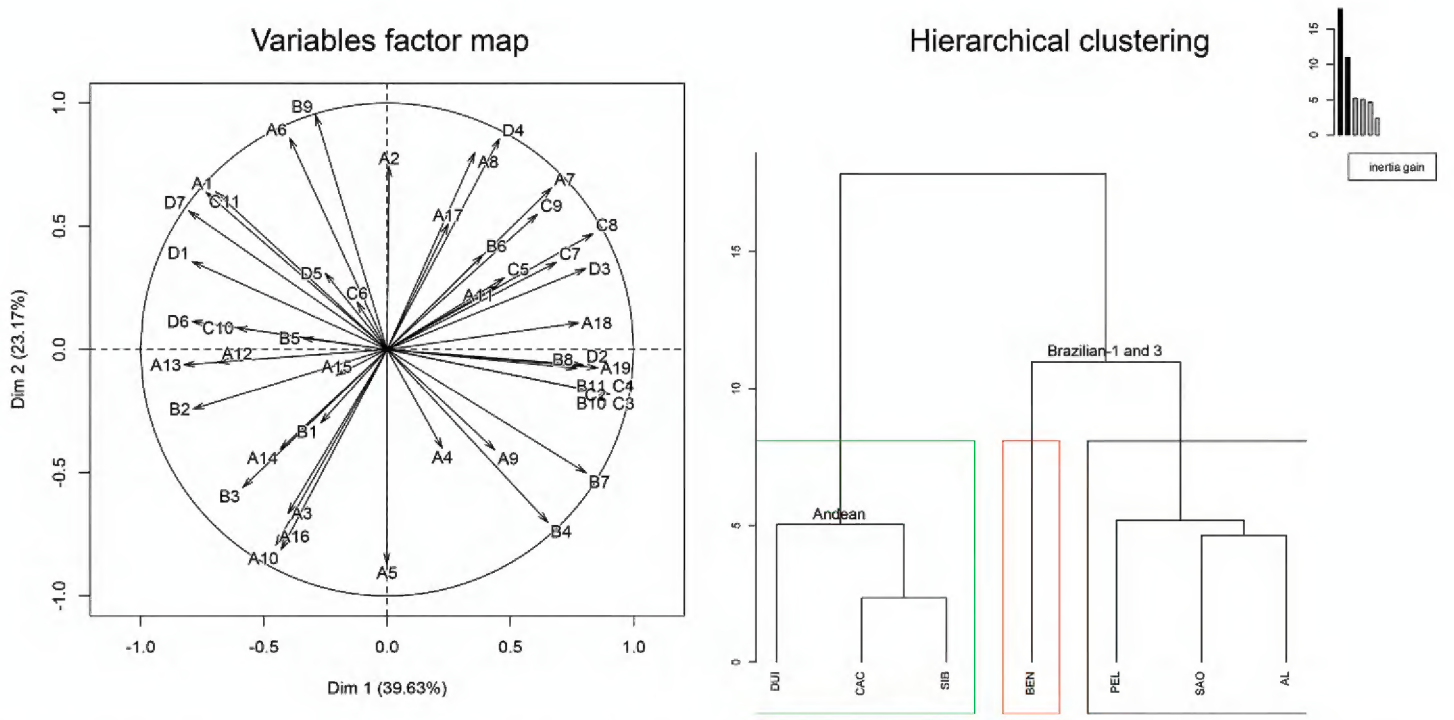


**Figure 4.** Principal component analyses (PCA) of transformed GC×GC/MS data of 48 female CHs from seven populations of the *Anastrepha fraterculus* cryptic species complex. (Variables factor map) projection of variables on the plane defined by the first two principal components. (Hierarchical clustering) score plot describing the populations and their clustering. Key: AL – Alagoas, AL, Brazil; BEN – Bento Gonçalves, RS, Brazil; CAC – Cachipay, Colombia; DUI – Duitama, Colombia; PEL – Pelotas, RS, Brazil; SAO – São Joaquim, SC, Brazil; SIB – Sibundoy, Colombia. A1–19 – *n*-alkanes; B1–11 – methyl-branched hydrocarbons; C1–11 – alkenes; D1–7 – alkadienes. Colored boxes indicate particular clusters.

unsaturated CHs (hentriacontene C8, tetratriacontadiene D4), which were specific to the Brazilian morphotypes. The Andean morphotype (CAC, DUI, SIB) was defined by the presence of linear compounds (*n*-dodecane A1, *n*-pentacosane A10, *n*-hentriacontane A16), a mix of methylbranched CHs (9-/11-/13-methylhentriacontane B9) and heptatriacontadiene (D7) (Figure 4, Variables factor map, Suppl. material 1).

## Discussion

Chemical profiles of *A. fraterculus* varied quantitatively among populations from diverse regions of South America. To some extent, the chemical profiles showed comparable patterns among populations belonging to the same morphotype. Comparison of the chemical profiles of the Andean and two Brazilian morphotypes showed that the more geographically distant the morphotypes are, the more diverse their pheromone and CH profiles are. Nevertheless, this trend was not observed between the Brazilian-1 and Brazilian-3 morphotypes. Within the *A. fraterculus* complex, the Andean morphotype is allopatric, while the Brazilian morphotypes (Brazilin-1, Brazilian-2 and Brazilian-3) are sympatric (Hernández-Ortiz et al. 2012, Selivon et al. 2005). The sympatry of the Brazilian entities may be one of the possible factors contributing to the similarity of their chemical profiles. Evidence of similarities of pheromone profiles within and between the species complexes of sympatric populations comes from an extensive evo-

lutionary study of Hawaiian Drosophilidae CHs (Alves et al. 2010). Such evolutionary studies are missing for the *A. fraterculus* complex and nothing is known about the role of pheromones (short- or long-range) in the speciation process of this species.

The Andean morphotype populations had very different pheromone profiles from that of the two Brazilian morphotypes. Břízová et al. (2013) and Cáceres et al. (2009) reported extensive qualitative and quantitative differences in the male pheromone composition of Brazilian, Argentinean and Peruvian populations of the *A. fraterculus* complex. Our study demonstrated that the common plant monoterpene, limonene, varies least among all populations. Two of the most variable volatiles from the male pheromone mixture were (*E*,*Z*)-3,6-nonadien-1-ol and (*E*,*E*)-α-farnesene. As reported by Milet-Pinheiro et al. (2015), (*E*,*Z*)-3,6-nonadien-1-ol, α-farnesene, and epianastrephin are highly attractive to conspecific females after approaching a mating site, while plant compounds such as α-pinene or limonene are used by female to find mating and brood sites (Robacker and Hart 1986). We speculate that the different ratios of (*E*,*Z*)-3,6-nonadien-1-ol, (*E*,*E*)-α-farnesene, and epianastrephin, together with the diverse CH profiles, result in the final rejection of a male by an heterospecific female. A recent study on reproductive compatibility demonstrated that the Andean morphotype is fully incompatible with the Brazilian-1 and Brazilian-3 morphotypes (Devescovi et al. 2014). The authors stated that prezygotic sexual incompatibility might be a result of the differences in the timing of mating activities between the morphotypes studied. Considering the mating of *A. fraterculus*, where chemical communication plays an important role in acceptance or rejection of males, we suggest that the differences in the chemical profiles identified in the present study may also contribute to sexual incompatibility. Nevertheless, further electrophysiological and behavioural studies involving different morphotypes of the *A. fraterculus* complex need to be performed in order to evaluate this hypothesis.

Male and female flies of the Andean morphotype and the Brazilian morphotypes can be separated using CH profiles (Vaníčková et al. in press). The Andean morphotype formes a separated group whereas the Brazilian-1 and Brazilian-3 morphotypes create a monophyletic cluster. Nonetheless, Brazilian populations belonging to the same geographical areas do not group together. Variation in the chemical composition of CH profiles identified here may be influenced by genetic variability within and between populations of the Brazilian-1 and 3 morphotypes. It is important to note that all of the Brazilian populations investigated here were created during several generations under identical laboratory conditions, which could possibly influence the results presented here. Houot et al. (2010) reported the effects of laboratory acclimation on the variation of male courtship, mating and the production of sex pheromone, in *D. melanogaster*. These authors concluded that the reproduction-related characters could diverge between neighboring *D. melanogaster* populations, and differently adapt to stable laboratory conditions. Nevertheless, in tephritidae these kinds of studies are missing.

Selivon et al. (2004) found conspicuous differences between sex chromosomes that separated the Brazilian-3 from the Brazilian-1 and Brazilian-2 morphotypes. The possibility for hybridization between distinct cryptic species within the *A. fraterculus* complex and meiotic recombination of chromosomal markers could form the genetic

basis by which CHs vary between related putative species. The evidence comes from experiments with *Drosophila* spp. that explained interspecific variation of CH profiles (Coyne et al. 1994, Coyne and Oyama 1995, Doi et al. 1996, Coyne and Charlesworth 1997). Future genetic analyses are necessary for evaluation of this hypothesis within the *A. fraterculus* complex.

CHs in insects serve primarily to prevent desiccation by reducing water loss (Blomquist et al. 1987, Blomquist and Bagnères 2010). Populations living in warmer and drier environments lose water less rapidly and usually have longer chain-length CHs than populations in humid habitats (Blomquist and Bagnères 2010). However, the relationship between CH structure and the capacity to resist desiccation is not so simple. In our previous work on CH profiles from geographically distinct populations of the *A. fraterculus* complex, we reported that the relative proportions of these compounds vary, depending on relative humidity, relative temperature and altitude (Vaníčková et al. in press). In the present study, CH profiles from the Andean morphotype formed one single cluster. These populations live naturally at high altitudes with lower relative temperatures, and therefore their specific CHs are long methylbranched compounds (e.g. methylheptatriacontane). However, the same conclusion is not possible to draw for the Brazilian-1 and Brazilian-3 morphotypes studied. The three Brazilian morphotypes may co-occur in the same localities infesting guava fruits (*Psidium guajava* L.) (Selivon et al. 2004). The Brazilian-3 morphotype seems to be restricted to the Atlantic coastal areas and may co-occur with the Brazilian-2 morphotype infesting guava and tropical almond (*Terminalia catappa* L.). The Brazilian-1 morphotype occurs from northern Argentina through southern and southeastern Brazil and may also co-occur together with the Brazilian-2 morphotype infesting guavas and oranges (*Citrus* sp.). In Brazil there are 70 different host plants for the *A. fraterculus* complex recorded (Zucchi 2007). Etges and Jackson (2001) reported that the variation of CH profiles between closely related species or between populations of these species of *D. mojavensis*, reflects the adaptation to different host plants. In these flies, the ratio of the principal CHs rapidly changed with laboratory acclimation, and influenced courtship and mating (Stennett and Etges 1997, Houot et al. 2010). These CH changes depend on enzymes whose level could represent a metabolic adaptation to host-plant chemicals (Higa and Fuyama 1993, Jones 2001, Houot et al. 2010). Varying the availability of different nutrients could also account for CH variation between strains raised in the laboratory (Stennett and Etges 1997). These factors, possibly influencing CH composition in the *A. fraterculus* complex, need to be carefully investigated in future studies.

## Conclusion

The present study demonstrates that pheromone components and CH profiles diverge qualitatively between Andean and Brazilian-1 and Brazilian-3 morphotypes and may be used to some extent to delimit morphotypes in the *A. fraterculus* species complex. Comprehensive studies, which simultaneously examine environmental, behavioural,

genetic and chemical features are necessary to be performed aiming to understand which factors affect the geographical variation in the male-borne volatiles and CH profiles in the *A. fraterculus* complex.

## Acknowledgements

The authors acknowledge Dr. Tara Massad for the language corrections and valuable comments, and the anonymous reviewers for their comments on the early version of the manuscript. Financial support was provided by the Institute of Organic Chemistry and Biochemistry, Academy of Science of Czech Republic, Prague (RVO: 61388963) and the Conselho Nacional de Desenvolvimento Científico e Tecnologico, Brazil (CNPq 376348/2012-7). The authors sincerely acknowledge support provided by the FAO/IAEA via the Coordinated Research Project 'Resolution of cryptic species complexes of tephritid pests to overcome constraints to SIT application and international trade'.

## References

Aluja M (1994) Binomics and management of *Anastrepha*. Annual Review of Entomology 39: 155–178. doi: 10.1146/annurev.en.39.010194.001103

Alves H, Rouault J-D, Kondoh Y, Nakano Y, Yamamoto D, Kim Y-K, Jallon J-M (2010) Evolution of cuticular hydrocarbons of Hawaiian Drosophilidae. Behavior Genetics 40: 694–705. doi: 10.1007/s10519-010-9364-y

Blomquist GJ, Dillwith JW, Adams TS (1987) Biosynthesis and endocrine reguation of sex pheromone production in Diptera. In: Prestwitch GD, Blomquist GJ (Eds) Pheromone Biochemistry. Academic Press, London, 217–250.

Blomquist GJ, Bagnères AG (2010) Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology. Cambridge University Press, New York. doi: 10.1017/CBO9780511711909

Břízová R, Mendonça AL, Vaníčková L, Lima-Mendonça A, da Silva CE, Tomčala A, Paranhos BAJ, Dias VS, Joachim-Bravo IS, Hoskovec M, Kalinová B, do Nascimento RR (2013) Pheromone analyses of the *Anastrepha fraterculus* (Diptera: Tephritidae) cryptic species complex. Florida Entomologist 96: 1107–1115. doi: 10.1653/024.096.0351

Cáceres C, Segura DF, Vera MT, Wornoayporn V, Cladera JL, Teal P, Sapountzis P, Bourtzis P, Zacharopoulou A, Robinson AS (2009) Incipient speciation revealed in *Anastrepha fraterculus* (Diptera; Tephritidae) by studies on mating compatibility, sex pheromones, hybridization, and cytology. Biological Journal of the Linnean Society 97: 152–165. doi: 10.1111/j.1095-8312.2008.01193.x

Carlson DA, Yocom SR (1986) Cuticular hydrocarbons from six species of Tephritid fruit flies. Archives of Insect Biochemistry and Physiology 3: 397–412. doi: 10.1002/arch.940030407

Coyne JA, Crittenden AP, Maht K (1994) Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. Science 265: 1461–1464. doi: 10.1126/science.8073292

Coyne JA, Oyama R (1995) Localization of pheromonal sexual dimorphism in *Drosophila melanogaster* and its effect on sexual isolation. Evolution 92: 9505–9509. doi: 10.1073/pnas.92.21.9505

Coyne JA, Charlesworth B (1997) Genetics of a pheromonal difference afecting sexual isolation between *Drosophila mauritianu* and *D. sechellia*. Genetics 145: 1015–1030.

Devescovi F, Abraham S, Roriz AKP, Nolazco N, Castañeda R, Tadeo E, Caceres C, Segura DF, Vera MT, Joachim-Bravo I, Canal N, Rull J (2014) Ongoing speciation within the *Anastrepha fraterculus* cryptic species complex: the case of the Andean morphotype. Entomologia Experimentalis et Applicata 152: 238–247. doi: 10.1111/eea.12219

Doi M, Tomaru M, Matsubayashi H, Yamanoi K, Oguma Y (1996) Genetic analysis of *Drosophila virilis* sex pheromone: genetic mapping of the locus producing (*Z*)-11-pentacosene. Genetic Research 68: 17–21. doi: 10.1017/S001667230003384X

Etges WJ, Jackson LL (2001) Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis* part 6 – Epicuticular hydrocarbon variation in *Drosophila mojavensis* cluster species. Journal of Chemical Ecology 27: 2125–2149. doi: 10.1023/A:1012203222876

Gemeno C, Laserna N, Riba M, Valls J, Castañé C, Alomar O (2012) Cuticular hydrocarbons discriminate cryptic *Macrolophus* species (Hemiptera: Miridae). Bulletin of Entomological Research 102: 624–631. doi: 10.1017/s0007485312000193

Haverty MI, Woodrow RJ, Nelson LJ, Grace JK (2000) Cuticular hydrocarbons of termites of the Hawaiian Islands. Journal of Chemical Ecology 26: 1167–1191. doi: 10.1023/A:1005479826651

Hernández-Ortiz V, Gomez-Anaya JA, Sánchez A, McPheron BA, Aluja M (2004) Morphometric analysis of Mexican and South American populations of the *Anastrepha fraterculus* complex (Diptera: Tephritidae) and recognition of a distinct Mexican morphotype. Bulletin of Entomological Research 94: 487–499. doi: 10.1079/BER2004325

Hernández-Ortiz V, Bartolucci AF, Morales-Valles P, Frías D, Selivon D (2012) Cryptic species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae): a multivariate approach for the recognition of South American morphotypes. Annals of the Entomological Society of America 105: 305–318. doi: 10.1603/an11123

Hernández-Ortiz V, Canal NA, Tigrero Salas JO, Ruíz-Hurtado FM, Dzul-Cauich JF (2015) Taxonomy and phenotypic relationships of the *Anastrepha fraterculus* complex in the Mesoamerican and Pacific Neotropical dominions (Diptera, Tephritidae). In: De Meyer M, Clarke AR, Vera MT, Hendrichs J (Eds) Resolution of Cryptic Species Complexes of Tephritid Pests to Enhance SIT Application and Facilitate International Trade. ZooKeys 540: 95–124. doi: 10.3897/zookeys.540.6027

Higa I, Fuyama Y (1993) Genetics of food preference in *Drosophila sechellia*. 1. Responses to food attractants 88: 129–136.

Houot B, Svetec N, Godoy-Herrera R, Ferveur J-F (2010) Effect of laboratory acclimation on the variation of reproduction-related characters in *Drosophila melanogaster*. The Journal of Experimental Biology 213: 2322–2331. doi: 10.1242/jeb.041566

Husson F, Josse J, Le S, Mazet J (2015) FactoMineR: Multivariate Exploratory Data Analysis and Data Mining. R package version 1.29. http://CRAN.R-project.org/package=FactoMineR

Jallon JM (1984) A few chemical words exchanged by *Drosophila* during courtship and mating. Behavior Genetics 14: 441–478. doi: 10.1007/BF01065444

Jennings JH, Etges WJ, Schmitt T, Hoikkala A (2014) Cuticular hydrocarbons of *Drosophila montana*: Geographic variation, sexual dimorphism and potential roles as pheromones. Journal of Insect Physiology 61: 16–24. doi: 10.1016/j.jinsphys.2013.12.004

Jones CD (2001) The genetic basis of larval resistance to a host plant toxin in *Drosophila sechellia*. Genetic Research 78: 225–233. doi: 10.1017/S0016672301005298

Kather R, Martin SJ (2012) Cuticular hydrocarbon profiles as a taxonomic tool: advantages, limitations and technical aspects. Physiological Entomology 37: 25–32. doi: 10.1111/j.1365-3032.2011.00826.x

Key M (2012) A tutorial in displaying mass spectrometry-based proteomic data using heat maps. BMC Bioinformatics 13 (Suppl 16): S10. doi: 10.1186/1471-2105-13-S16-S10

Kroiss J (2008) Chemical attraction and deception. Intra- and interspecific communication in Hymenoptera. PhD thesis, Universität Regensburg, Regensburg, Germany.

Levine JD, Millar JG (2009) Chemical signalling: laser on the fly reveals a new chemical word. Current Biology 19: R653–R655. doi: 10.1016/j.cub.2009.06.051

Lima IS, House PE, do Nascimento RR (2001) Volatile substances from male *Anastrepha fraterculus* Wied. (Diptera: Tephritidae): Identification and behavioural activity. Journal of Brazilian Chemical Society 12: 196–201. doi: 10.1590/S0103-50532001000200012

Lima-Mendonça A, Mendonça AdL, Sant´Ana AEG, Do Nascimento RR (2014) Semioquímicos de moscas das frutas do genero *Anastrepha*. Química Nova 37: 293–301.

Milet-Pinheiro P, Navarro DMA, De Aquino NC, Ferreira LL, Tavares RF, Da Silva RCC, Lima-Mendonça A, Vaníčková L, Mendonça AL, Do Nascimento RR (2015) Identification of male-borne attractants in *Anastrepha fraterculus* (Diptera: Tephritidae). Chemoecology 25: 115–122. doi: 10.1007/s00049-014-0180-3

Millar JG (2000) Polyene hydrocarbons and epoxides: a second major class of lepidopteran sex attractant pheromones. Annual Review of Entomology 45: 575–604. doi: 10.1146/annurev.ento.45.1.575

Morgante JS, Malavasi A, Bush GL (1980) Biochemical systematics and evolutionary relationships of neotropical *Anastrepha*. Annals of the Entomological Society of America 73: 622–630 doi: 10.1093/aesa/73.6.622

Morgante JS, Malavasi A, Prokopy RJ (1983) Mating behavior of wild *Anastrepha fraterculus* (Diptera: Tephritidae) on a caged host tree. Florida Entomologist 66: 234–241. doi: 10.2307/3494246

Oliveira C, Manfrin MH, Sene F, Jackson LL, Etges WJ (2011) Variations on a theme: diversification of cuticular hydrocarbons in a clade of cactophilic *Drosophila*. BMC Evolutionary Biology 11: 1–19. doi: 10.1186/1471-2148-11-179

R Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org

Robacker DC, Hart WG (1986) Behavioral response of male and female Mexican fruit flies, *Anastrepha ludens*, to male-produced chemicals in laboratory experiments. Journal of Chemical Ecology 12: 39–47. doi: 10.1007/BF01045589

Rull J, Abraham S, Kovaleski A, Segura DF, Mendoza M, Liendo MC, Vera MT (2013) Evolution of pre-zygotic and post-zygotic barriers to gene flow among three cryptic species within the *Anastrepha fraterculus* complex. Entomologia Experimentalis et Applicata 148: 213–222. doi: 10.1111/eea.12094

Segura DF, Vera MT, Rull J, Wornoayporn V, Ismal A, Robinson AS (2011) Assortative mating among *Anastrepha fraterculus* (Diptera: Tephritidae) hybrids as a possible route to radiation of the fraterculus cryptic species complex. Biological Journal of the Linnean Society 102: 346–354. doi: 10.1111/j.1095-8312.2010.01590.x

Selivon D, Vretos C, Fontes L, Perondini ALP (2004) New variant forms in the *Anastrepha fraterculus* complex (Diptera, Tephritidae). 6th International Symposium on Fruit Flies of Economic Importance, 257–258.

Selivon D, Perondini ALP, Morgante JS (2005) A genetic–morphological characterization of two cryptic species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae). Genetics 98: 367–381. doi: 10.1603/0013-8746(2005)098[0367:agcotc]2.0.co;2

Sobrinho RB, Guimarães JA, Mesquita ALM, Araújo KLB (2009) Desenvolvimento de dietas para a criação massal de mosca-das-frutas do gênero *Anastrepha* (Diptera:Tephritidae). Boletim de Pequisa e Desenvolvimento da EMBRAPA 33: 12–14.

Stennett MD, Etges WJ (1997) Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. III. Epicuticular hydrocarbon variation is determined by use of different host plants in *Drosophila mojavensis* and *Drosophila arizonae*. Journal of Chemical Ecology 23: 2803–2824. doi: 10.1023/A:1022519228346

Van Den Dool H, Kratz PD (1963) A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatograpy. Journal of Chromatograpy A 11: 463–471. doi: 10.1016/S0021-9673(01)80947-X

Vaníčková L (2012) Chemical ecology of fruit flies: genera *Ceratitis* and *Anastrepha*. PhD thesis, Institute of Chemical Technology, Prague, Czech Republic.

Vaníčková L, do Nascimento RR, Hoskovec M, Ježková Z, Břízová R, Tomčala A, Kalinová B (2012a) Are the wild and laboratory insect populations different in semiochemical emission? The case of medfly sex pheromone. Journal of Agricultural and Food Chemistry 60: 7168–7176. doi: 10.1021/jf301474d

Vaníčková L, Svatoš A, Kroiss J, Kaltenpoth M, Nascimento RR, Hoskovec M, Břízová R, Kalinová B (2012b) Cuticular hydrocarbons of the South American fruit fly *Anastrepha fraterculus*: variability with sex and age. Journal of Chemical Ecology 38: 1133–1142. doi: 10.1007/s10886-012-0177-8

Vaníčková L, Virgilio M, Tomčala A, Břízová R, Ekesi S, Hoskovec M, Kalinová B, Do Nascimento RR, De Meyer M (2014) Resolution of three cryptic agricultural pests (*Ceratitis fasciventris*, *C. anonae*, *C. rosa*, Diptera: Tephritidae) using cuticular hydrocarbon profiling. Bulletin of Entomological Research 104: 631–638. doi:10.1017/S0007485314000406

Vaníčková L, Hernández-Ortiz V, Bravo ISJ, Dias V, Roriz AKP, Laumann RA, Mendonça AL, Paranhos BAJ, do Nascimento RR (2015) Current knowledge of the species complex *Anastrepha fraterculus* (Diptera, Tephritidae) in Brazil. In: De Meyer M, Clarke AR, Vera MT, Hendrichs J (Eds) Resolution of Cryptic Species Complexes of Tephritid Pests to

Enhance SIT Application and Facilitate International Trade. ZooKeys 540: 211–237. doi: 10.3897/zookeys.540.9791

Vaníčková L, Břízová R, Pompeiano A, Ekesi S, De Meyer M (2015) Cuticular hydrocarbons corroborate the distinction between lowland and highland Natal fruit fly (Tephritidae, *Ceratitis rosa*) populations. In: De Meyer M, Clarke AR, Vera MT, Hendrichs J (Eds) Resolution of Cryptic Species Complexes of Tephritid Pests to Enhance SIT Application and Facilitate International Trade. ZooKeys 540: 507–524. doi: 10.3897/zookeys.540.9619

Vaníčková L, Břízová R, Mendonça AL, Pompeiano A, Do Nascimento RR (in press) Intraspecific variation of cuticular hydrocarbon profiles in the *Anastrepha fraterculus* (Diptera: Tephritidae) species complex. Journal of Applied Entomology. doi: 10.1111/jen.12204

Vera MT, Cáceres C, Wornoayporn V, Islam A, Robinson AS, De La Vega MH, Hendrichs J, Cayol JP (2006) Mating incompatibility among populations of the South American fruit fly *Anastrepha fraterculus* (Diptera: Tephritidae). Annals of the Entomological Society of America 99: 387–397. doi: 10.1603/0013-8746(2006)099[0387:MIAPOT]2.0.CO

Warnes GR, Bolker B, Bonebakker L, Gentleman R, Liaw WHA, Lumley T, Maechler M, Magnusson A, Moeller S, Schwartz M, Venables B (2015) gplots: Various R Programming Tools for Plotting Data. R package version 2.16.0. http://CRAN.R-project.org/package=gplots

Zucchi RA (2007) Diversidad, distribution y hospederos del género *Anastrepha* en Brasil. In: Hernández-Oritz V (Ed.) Mosca de la Fruta en Latinoamérica (Diptera: Tephritidae): Diversidad, Biología y Manejo. Distrito Federal, México, 77–100.

## Supplementary material 1

**Table S1. *Anastrepha fraterculus* male and female characteristic.**

Authors: Lucie Vaníčková, Radka Břízová, Antonio Pompeiano, Luana Lima Ferreira, Nathaly Costa de Aquino, Raphael de Farias Tavares, Laura D. Rodriguez, Adriana de Lima Mendonça, Nelson Augusto Canal, Ruth Rufino do Nascimento

Data type: species data

Explanation note: *Anastrepha fraterculus* male (m) and female (f) characteristic cuticular hydrocarbons identified by principal component analyses.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.